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C. Jaing, J. Thissen, S. Gardner, K. McLoughlin, P. Hullinger, N. Monday, M. Niederwerder, R. Rowland

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The application of a pathogen microarray for the analysis of viruses and bacteria in clinical diagnostic samples from pigs

Microarray detection of pig pathogens

Crystal J. Jaing*[1], James B. Thissen[1], Shea Gardner[2], Kevin McLoughlin[2], Pam Hullinger[1], Nicholas Monday[3], Megan Niederwerder[3], Raymond R.R. Rowland[3]
[1]Physical & Life Sciences Directorate, [2]Computations Directorate, Lawrence Livermore National Laboratory, Livermore, CA, [3]Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS

*Corresponding author:
Crystal Jaing
jaing2@llnl.gov
Lawrence Livermore National Laboratory
P.O. Box 808
Mailstop L-452
Livermore, CA 94551

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Abstract:
Many of the disease challenges of a modern swine industry involve the analysis of complex problems caused by polymicrobial, emerging/re-emerging and foreign disease pathogens. The Lawrence Livermore Microbial Detection Array is designed to detect up to 8,101 species of microbes. This study evaluated the utility of the array to evaluate the microbial composition of veterinary diagnostic samples, including serum, oral fluid and tonsil. Samples were obtained from a population of pigs co-infected with porcine reproductive and respiratory syndrome virus and porcine circovirus type 2, two endemic pathogens linked to a variety of polymicrobial syndromes. Under conditions of experimental infection, the array identified porcine reproductive and respiratory syndrome virus and porcine circovirus type 2, but at a lower sensitivity compared to standard polymerase chain reaction detection methods. The pen-based oral fluid sample was the most informative, possessing signatures from several porcine-associated viruses and bacteria, which may contribute to the severity of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 diseases.

Keywords:
Bacteria, diagnostics, disease, microarray, microbial, pathogen, pig, virus

Introduction

Agriculture accounts for $1.24 trillion, or 12.3 percent, of the Gross Domestic Product in the United States. Any deliberate or natural disruptions resulting from the introduction of infectious diseases can produce enormous impacts that affect domestic production, consumption, exports, and for zoonotic pathogens, public health. Recent examples include the introduction of influenza H1N1 and porcine epidemic diarrheal virus (PEDV) in swine.[1,2] Currently circulating foreign animal diseases, such as African swine fever and classical swine fever are constant threats and raise concern about the possibility of both intentional as well an unintentional introduction. The best assurance of timely identification for known and unknown threats is to employ techniques that can track known disease threats, as well as rapidly identify the introduction of new pathogens before they become established.

PCR and DNA sequencing are widely used for diagnostic pathogen detection and characterization. PCR assays are limited, in that only a single or few organisms can be investigated per assay. While DNA sequencing can identify a broader scope of organisms, current DNA sequencing analysis methods are lengthy, costly and require significant expertise and computational time. Sensitivity for viruses with small genomes may be low, as their signal is swamped by host and bacterial reads. An alternative approach is the use of microarrays, which probe a sample for nucleotide signatures from known agents. The Lawrence Livermore Microbial Detection Array (LLMDA) was developed to probe for all known microbiological agents for which whole genomes are available. The most recent version [3] contains probes to detect 8,101 species of microbes including 3,856 viruses, 3,855 bacteria, 254 archaebacteria, 100 fungi, and 36 protozoa (sequenced through June, 2013). The microarray targets both conserved and unique genomic regions of sequenced microbial strains. The automated data analysis algorithm, Composite Likelihood Maximization, is integrated with a web interface that enables LLMDA data analysis within 30 minutes.

The application of array technology has primarily focused on the discovery of previously sequenced agents responsible for undiagnosed syndromes. As the cost of array technology continues to decrease and throughput increases, it is now possible to include array screening of routine diagnostic samples. In this study we leveraged experimental infection of pigs with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type-2 (PCV2) to investigate the application of the LLMDA for use in the analysis of clinical samples. Both viruses are endemic in pig populations globally and create an environment inside the pig that supports several polymicrobial syndromes, including porcine respiratory disease complex (PRDC) and porcine circovirus-associated disease (PCVAD). [4-6]

In veterinary diagnostics, serum is a standard sample for evaluating the presence of a variety of pathogens. One alternative to serum is oral fluid, which is typically collected by allowing pigs to chew on a rope in a single pen. The oral fluid contents include serum components, which leak out of capillary beds that line the buccal cavity. Advantages to oral fluid over serum include, 1) ease of collection by animal caretakers, 2) the relative non-invasive nature of the collection procedure, 3) collection of samples on an as-needed basis, and 4) the collection of a "pooled" sample for increased coverage in large commercial swine operations. Potential drawbacks include dilution of the sample contributed by several pigs and sick animals may not chew on the rope or the pathogen is not shed in oral fluids, and hence the pathogen could go undetected. However, Olsen et al. demonstrated that in some cases, oral fluid sampling can detect the introduction of a virus sooner than standard serum collection techniques. [7]

The purpose of this study was to compare the LLMDA for use in serum, oral fluid and tonsil samples from pigs co-infected with PRRSV and PCV2. The assay was compared to standard PCR methods for the detection of PRRSV, an RNA virus and PCV2, a DNA virus. Since PRRSV and PCV2 are immunosuppressive, a second goal was to evaluate the presence of other agents that may contribute to disease.

Materials and methods

Pigs and viruses. All experiments involving animals and viruses were approved by the Kansas State University institutional animal care and biosafety committees as part of a study on host genetics associated with PRRS. The PRRSV/PCV2 inoculum used for infection of pigs was prepared from a lymph node derived from a pig with severe postweaning multisystemic wasting syndrome (PMWS) and was described in detail previously. [8] Since PCV2b does not propagate to high levels in cell culture, we took advantage of the heat stability of PCV2 to make a virus preparation from a lymph node suspension enriched for PCV2. The suspension was heat-treated at 55^{o} for 30 min followed by filtration through a 0.2 µm filter to remove heat-labile viruses and bacteria. Analysis of the heat-treated preparation for common agents showed that the preparation was negative for other heat stable agents, such as parvovirus. The resulting treatments resulted in 10^{8} $TCID_{50}$ of PCV2 in each mL of filtered homogenate. A 1:100 dilution was used in the inoculum. Prior to heat treatment, virus isolation for PRRSV was performed on MARC-145 cells, a simian cell line. The PCV2 homogenate

and PRRSV were combined to yield a final concentration of 10^5 $TCID_{50}$/mL for both viruses. The experimental design included the infection of 200 pigs, 7 weeks of age. Each pig was challenged with 1 mL intranasally and 1 mL intramuscularly of viral inoculum. Three weeks prior, one half of the pigs were vaccinated with a commercial modified live virus (MLV) vaccine according to the label instructions (GenBank Accession #AF159149). Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 35, and 42 days post-infection (dpi). Oral fluid was collected by suspending a 1.25 cm diameter twisted cotton rope in a pen of pigs. Prior to the experiment, pigs were conditioned to chew on the rope. For oral fluid collection, the rope was suspended at about shoulder high for the pigs and pigs were allowed to actively chew on the rope for approximately 30 minutes. The rope was removed, placed in a sealed plastic bag, fluid contents extracted with a double roller wringer and stored at -80^{o}C prior to use.

PRRSV and PCV2 PCR. **V**iral DNA and RNA were extracted simultaneously from serum and oral fluid samples using Ambion's MagMAX™ 96 Viral Isolation Kit (Applied Biosystems®, Foster City, CA) in accordance to the manufacturer's instructions. Viral DNA was extracted from tonsils using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for tissue. PRRSV RNA was quantified using EZ-PRRSV™ MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore®, Rockville, MD) according to the manufacturer's instructions. For consistency, each plate contained Tetracore® Quantification Standards and Control Sets for use with EZ-PRRSV™ MPX 4.0 RT-PCR Reagents. All PCR reactions were carried out on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) in a 96-well format using the recommended cycling parameters. PCV2 DNA was quantified using an SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad). PCV2 DNA was amplified using the forward primer 5'-AATGCAGAGGCGTGATTGGA-3' and reverse primer 5'-CCAGTATGTGGTTTCCGGGT-3'. Primers were used at a final concentration of 300 µM in a 20 µL reaction. Positive and negative controls were included on each plate. Plasmid DNA with a PCV2 sequence (a field strain PCV2b 321/393) was used for the PCV2 standard curve and positive control. Plasmid DNA was isolated using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI). The DNA was quantified using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The standard curve for this assay was performed by diluting the purified plasmid DNA 1:1000 in nuclease free water followed by five serial 1:10 dilutions. The final standard curve possessed 6 points ranging from approximately 10^2 to 10^7 genome copies of template, which produced threshold values between 15 and 33 cycles in the PCR reaction. Standard curves were run in duplicate with nuclease-free water as a negative control. The PCV2 PCR was carried out using the following settings: activation at 98°C for 2 minutes, followed by 40 cycles of denaturing at 98°C for 5 seconds and annealing/extension at 60°C for 10 seconds. The melting curve was performed between 65-95°C using 0.5°C increments. The assay results were reported as $\log_{10}$ PRRSV RNA starting quantity (copy number) or PCV2 DNA starting quantity per reaction.

Microarray analysis. Each extracted sample possessed a volume of 1 mL, which consisted of 250 µL sample and 750 µL of Trizol LS reagent. Each sample was brought to room temperature, 200 µL of chloroform was added, and the tube was shaken vigorously for 15 seconds. Samples were incubated at room temperature for 15 min and then centrifuged at 12,000xg for 15 min at 4°C. The upper aqueous layer was removed by pipetting and placed in a new tube for RNA extraction. The lower phases were saved for DNA extraction**.** For RNA extraction, 10 µg of glycogen was added to the aqueous phase along with 500 µL of 100% isopropanol. Following 10 min incubation, samples were centrifuged for 10 minutes at 12,000xg and 4°C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol. The sample was vortexed and centrifuged at 7500xg for 5 minutes at 4°C. Following centrifugation, the supernatant was removed and the RNA was air dried for 10 min. RNA pellets were re-suspended in DEPC water and RNA concentration was determined by Nanodrop spectrophotometer and a Qubit fluorometer.

For DNA isolation, 300 µL of 100% ethanol was added to the interphase/organic phase; the tube was inverted several times, and incubated for 3 min at room temperature. Samples were centrifuged at 2000xg for 5 min at 4°C and the supernatant was discarded. The DNA pellet was washed with 1 mL sodium citrate/ethanol solution (0.1M sodium citrate in 10% ethanol, pH 8.5), incubated for 30 min at room temperature, centrifuged at 2000xg for 5 min at 4°C, and supernatant removed. The sodium citrate/ethanol wash procedure was repeated once more. Following the wash procedures, 2 mL of 75% ethanol was added to the pellet and incubated at room temperature for 20 min and centrifuged at 2000xg for 5 min at 4°C. The supernatant was removed and the samples were air dried for 10 min. DNA pellets were resuspended in 8 mM NaOH solution and the DNA concentration was determined by Nanodrop spectrophotometer and Qubit fluorometer.

For each sample, 5 µL (0.67 to 28.4 ng/µL) of extracted RNA was used as input into the random amplification procedure. The random amplification was initiated by mixing 5 µL of extracted RNA with 5 µL of water and 1 µL of random primer 5'-GATGAGGGAAGATGGGGNNNNNNNNN-3' (100 pmole/µL). This 10 µL mixture was then incubated for 2 min at 85^{o}C and immediately placed on ice for 2 min. Following ice incubation, each sample received 4 µL 5x Invitrogen Superscript III buffer, 1µL dNTP (12.5mM), 2 µL DTT (0.1M), 1 µL Invitrogen Superscript III reverse transcriptase, and 1 µL DEPC water (Invitrogen). Each reaction was subsequently placed in an MJ Tetrad thermocycler and cycled with the following conditions to achieve first strand cDNA synthesis: 25^{o}C for 10 min, 42^{o}C for 2 hr, and 70^{o}C for 5 min. After cycling completed, a second strand cDNA reaction was then carried out by adding 2.4 µL 10X Klenow buffer (New England Biolabs, Ipswich, MA) and 0.5 µL 12.5 mM dNTP to each 20 µL reaction and incubating at 85^{o}C for 2 min followed by incubation on ice for 2 min. Lastly, 1 µL of Klenow polymerase (New England Biolabs) was added to the samples and allowed to incubate at 37^{o}C for 60 min followed by 70^{o}C for 20 min.

To complete the amplification of the samples, 5 µL of the double-stranded cDNA reaction was mixed with 10 µL 5x Phusion HF buffer (New England Biolabs), 1 µL dNTP (10mM), 1 µL primer 5'-GATGAGGGAAGATGGGG-3' (100 pmole/µL), 0.5 µL Phusion DNA polymerase (New England Biolabs), and 32.5 µL water. This 50µL was then cycled in an MJ Tetrad thermocycler with the following conditions: 98^{o}C for 2 min, 35 cycles of [98^{o}C for 1 min, 60^{o}C for 1 min, 72^{o}C for 1 min], and 72^{o}C for 10 min. Amplified cDNA was purified using a Qiaquick PCR column (Qiagen, #28106). The amplified cDNA yield was 644-1580 ng as determined by Qubit fluorometer. For each DNA sample, 5 µL (0.036 to 0.90 ng/µL) was used as input into a RepliG Midi kit (Qiagen, #150043) using standard manufacturer's protocols. The amplified DNA was purified using a Qiaquick PCR columns and the yield was determined to be 5400-9200ng by Qubit fluorometer.

Approximately 400-500 ng of amplified cDNA and DNA was mixed together and labeled using the Roche NimbleGen One-Color Labeling kit (Roche, #06370411001) following standard manufacturer's protocols. The Agilent CGH hybridization mix (#5188-5220) was prepared following the standard manufacturer protocol with 10 µg of fluorescently labeled DNA added for each sample. Each labeled sample was mixed with the hybridization mastermix, denatured at 95°C for 3 min, and incubated at 65°C until the arrays were ready to load. The Agilent MDAv7 4x180K microarray was utilized for this work and samples were loaded onto the array and allowed to hybridize for 40 hr at 65°C in the Agilent rotator oven (#G2545A) set to rotation speed 20. After hybridization the microarrays were washed following standard manufacturer protocols with the Agilent CGH wash buffers (#5188-5226). Each array was washed for 1 minute in CGH Wash 1 at room temperature followed by 5 min in CGH Wash 2 at 37°C. After washing, the microarrays were exposed to a stream of nitrogen gas to remove any particulates from the array surface. Microarrays were scanned on the Roche NimbleGen MS200 scanner at a resolution of 2 µM.

Microarray data were analyzed using the composite likelihood maximization method developed at Lawrence Livermore National Laboratory. [3] The log likelihood for each of the possible targets is estimated from the BLAST similarity scores of the probe and target sequences, together with the probe sequence complexity and other covariates derived from the BLAST results. [3] Presented are the data for viral sequences that were equal to or greater than the 99% threshold.

Results

Analysis of the inoculum used for experimental infection of pigs. Pigs were infected with an inoculum derived from two sources: homogenized pig lymph node and the simian cell line, MARC-145. Since it was not possible to grow PCV2 to sufficient levels in culture, we incorporated a heat-treated and filtered homogenate from a diseased pig that possessed 10^8 $TCID_{50}$/mL of PCV2. As summarized in Table 1, the LLMDA analysis of the PCV2 inoculum identified PCV2 and signatures from two additional virus families. The first was torque teno sus virus (TTSuV), a member of a circular single-stranded DNA virus in the family, *Anelloviridae*. Signatures revealed two genera present in the sample, *Iotatorquevirus*, represented by TTSuV 1a and 1b, and

Kappatorquevirus, represented by TTSuV k2b. The second viruses identified were porcine type-C oncoviruses A and E. These viruses belong to a group collectively referred to as porcine endogenous retroviruses (PERVs). The analysis of the MARC-145 cell preparation identified PRRSV and a second virus, Mason−Pfizer simian endogenous retrovirus, a likely contaminant of the MARC-145 cell line.

Analysis of PCV2 and PRRSV in serum. Serum samples for analysis were obtained from pigs at 13 and 21 days after co-infection with PRRSV and PCV2. The selection of samples relative to the level of viremia is shown in Figure 1. Over the course of infection, PRRSV and PCV2 exhibited different kinetics. For PRRSV, mean viremia within the group of 200 pigs peaked at 7 days after infection and then declined until virus had disappeared from the blood of almost all pigs by 42 days after infection. In contrast, PCV2 viremia peaked at about 21 days and remained elevated during the remainder of the study period. The results, of selected sera, presented in Table 2, showed that the LLMDA easily detected PCV2 and PRRSV. For PCV2, serum samples with copy numbers equal to or greater than log 3.4 (Ct=28.2) were positive on the array. Similar results were obtained for PRRSV: serum samples with an estimated copy number equal to or greater than log 2.8 (Ct = 30.3) were positive on the array; whereas, all samples with a log copy number equal to or less than 2.6 (Ct=30.6) were negative for the microarray. Even though the data showed that PCR was more sensitive than the microarray for the detection of PRRSV and PCV2, the array was able to accurately detect the presence of both viruses when significant quantities of nucleic acid was present.

Besides PRRSV and PCV2, the array detected sequences from other porcine viruses. The most common were TTSuV and porcine type−C oncovirus, which were also present in the inoculum (Table 1). The Mason−Pfizer endogenous retrovirus sequence signatures detected in the MARC-145 cell preparation were not detected in serum or in any of the other samples. A signature for bocavirus 4-1 was identified in one sample, Serum 147. Signatures for three bacteria, D. acidovorans (samples160, 161, 165), M. hyopneumoniae (Sample 157) and S. maltophilia (samples 159, 161, 168) were also detected.

Analysis of oral fluid samples. Oral fluid is a multi-source sample derived from a group of pigs. The composition of oral fluid includes serum exudate from capillaries lining the buccal cavity, saliva, other oral cavity contents, etc. Additional animal sources include skin and feces. Another source of bacteria present on the rope comes from the environment, such as feed, water and surfaces. For this study, oral fluid samples were obtained from six pens (approximately 10 pigs per pen) at 13 days after co-infection, a time when significant amounts of PCV2 and PRRSV were present in serum (see Figure 1). PCV2/PRRSV PCR and microarray results are summarized in Table 3. The pathogen microarray identified the presence of PCV2 in all samples. Quantities of PCV2 DNA ranged between 4.3 and 5.6 log templates per reaction. All oral fluid samples were negative for PRRSV nucleic acid. Only one sample was PCR positive. The Ct value for the one positive sample was 38.6 or less than 1 log template per reaction. Signatures for a variety of viruses were identified, including TTSuV (6 of 6 samples), which was

also found in serum and the inoculum used for infection. In addition, array signatures revealed the presence of porcine stool-associated circular virus (6 of 6 samples), astrovirus (1 sample), dyodelta papillomavirus or Sus scrofa papillomavirus (1 sample) and porcine parainfluenza virus (1 sample). At a lower detection stringency (probe signal at 95% above random controls), astrovirus was detected in 3 additional samples (data not shown). All four viruses have been previously described in pigs[9-12].

Signatures related to 15 bacterial genera were identified. As summarized in Table 4, the predominate bacteria were *Streptococcus suis* (6 out of 6 samples), *Clostridiun sp*. (6 out of 6 samples), and *Staphyloccocus sp*. (6 out of 6 samples). Within this group, *S. suis* is an important swine pathogen and a disease cofactor associated with PRRSV and PCV2 infections. [13,14] The other bacterial pathogens identified were: *Enterococcus sp.* (4 out of 6 samples), *Psychrobacter sp.* (3 out of 6 samples), *Bibersteinia trehalosi* (3 out of 6 samples), *Aerococcus viridans* (3 out of 6 samples), *Actinobacillus pleuropneumoniae* (2 out of 6 samples) , *Lactobacillus sp*. (2 out of 6 samples), *Mycoplasma moatsii* (2 out of 6 samples), *Enhydrobacter aerosaccus* (2 out of 6 samples), *Bergeyella zoohelcum* (2 out of 6 samples), and *Lactobacillus* (1 out of 6 samples). The signature for a plasmid from *Haemophilus parasuis* was detected in one sample (data not shown).

Microarray and PCR analyses of tonsil. The results for tonsil, collected at termination or 42 days after infection, are summarized in Table 5. By 42 days after co-infection, PCV2 was still present at relatively high levels in the blood, but PRRSV had largely disappeared (see Figure 1). Out of the tonsil samples available for PCR testing, PCV2 was easily detected in all samples with values ranging between 1.6 and 6.1 log templates per reaction. Pathogen microarray detected all but one tonsil sample which had a PCR value equivalent to 1.6 log templates per reaction (Ct=34.4). At 42 days, the array failed to detect PRRSV. Only one tonsil was positive by PCR (Ct=36.0 or 0.9 log templates). Other virus signature detected in tonsil included TTSuV (3 of 12 tonsils) and porcine oncovirus (12 of 12 samples). *Actinobacillus pleuropneumoniae* was detected in two samples, *Pasturella multocida* plasmid sequence was found in one sample, and *Pasturella aerogenes* was found in another sample.

Discussion

Along with PCR and DNA sequencing, microarrays provide an efficient method for microbial detection and discovery. Even though microarrays are not as sensitive as standard PCR assays, they create the opportunity to query hundreds of thousands to several million sequence-specific DNA signatures, all in parallel.[3,15,16] As the cost of microarrays decrease, the application for use in routine diagnostics and disease surveillance in veterinary livestock is expected to increase, especially in the analysis of syndromes that result from polymicrobial interactions. The application of microarray technology in the veterinary diagnostic field creates several challenges, including: 1) attaining sufficient sensitivity to detect all relevant microbes, 2) validating positive results and eliminating false targets, and 3) integrating the results into good management decisions. In this study, we tested samples from a study using an

experimental infection model for the analysis of PCV2 and PRRSV in clinical samples. PRRSV and PCV2 are common pathogens in the commercial swine industry and participate as cofactors in a variety of polymicrobial disease syndromes.[4,17] This model system created the opportunity to validate the detection of known agents as well as evaluate the presence of other infectious agents. The starting point for the microarray analysis of clinical samples was the material used for the experimental infection of pigs. Besides the detection of signatures for PRRSV and PCV2, the microarray identified signatures of TTSuV, porcine oncoviruses, and a simian retrovirus. TTSuV and PERVs are commonly associated with pigs and were identified in the clinical samples.[18,19] The simian retrovirus was likely from the MARC-145 cell line used for propagation of PRRSV, but was not detected in any of the pig samples. The presence of TTSuV and porcine retroviruses suggests that the sources were from the lymph node used to prepare the PCV2 inoculum. This result emphasizes the importance for thorough analysis of virus preparations for the presence of adventitious agents, especially those preparations used for developing experimental animal models. However, in this case, both TTSuV and porcine retroviruses are already endogenous to pigs. PERVs are not considered significant pathogens of swine. How TTSuV functions as a pathogen remains controversial and under intense study. The strongest connection between TTSuV and disease is as a co-actor in PCV2-associated syndromes. [20]

Serum is a routine diagnostic sample used for the molecular detection of a variety of pathogens. The LLMDA easily identified PCV2 and PRRSV in sera from experimentally infected pigs (Table 2). Based on comparisons with standard PCR methods, the LLMDA was estimated to be about two orders of magnitude less sensitive. The level of sensitivity was consistent regardless of sample source. In samples such as tonsil and oral fluid, the absence of PRRSV detection by the LLMDA was due to insufficient PRRSV RNA. Therefore, the array is likely to deliver a positive result for these and other pathogens when relatively large quantities of microbe nucleic acid are present. Besides TTSuV and porcine oncoviruses, a porcine bocavirus signature was identified in a single serum sample (Table 2). Bocaviruses are members of the parvovirus family and represent a diverse group. A recent report by Huang et al [21] identified a high frequency of bocavirus sequences in samples from commercial operations. The role of bovaviruses as pathogens remains unknown. Signatures from three bacteria were identified in several serum samples (see Table 2). *M. hyopneumoniae* is an endemic pathogen of swine and a co-factor frequently associated with PRRSV and PCV2 infections. [17] The second bacterium, *S. maltophilia* is also associated with pigs. Its role as a pig pathogen is unknown, but is reported as a contaminant of extended pig semen. [22]

For disease surveillance at the population level, oral fluid has emerged as an important clinical specimen. The principal advantages are the ease of collection and the capacity to detect infectious diseases in pig populations sooner. [7,23,24] Theoretically, a small number of oral fluid samples can detect all of the microbes present in a population. As summarized in Table 3, PCV2 was easily detected by the LLMDA; however, all samples were negative for PRRSV. The inability to detect PRRSV was easily explained by the small quantity of PRRSV present in oral fluids, despite that we

tested samples collected at day 13, near the peak of viremia (Figure 1). As expected, signatures were detected for a variety of viruses and bacteria. In addition to pig-associated flora, oral fluid will likely contain microbes from environmental sources, such as feed, water, etc. Even though these bacteria may not participate in disease, they are sources of contamination of meat in the post-harvest processing. Besides TTSuV, several viral signatures were detected that represented feces-associated porcine viruses, including stool-associated circular virus 2 (PoSCV2) and a porcine astrovirus. Stool associated viruses were first described by the metagenomic analysis of fecal samples. [10] The roles of PoSCV2 as significant pathogens of swine remain unknown. Porcine astroviruses represent a diverse group, first characterized in in the 1980's. Since the most frequent source for isolation is fecal material, astroviruses have been linked to a variety of intestinal diseases.[11] The presence of feces-associated microbes in the oral fluid samples is likely the result of contamination by fecal material. Other viruses detected in oral fluid samples were dyodelta-papillomavirus (PV) 1 or sus scrofa papillomavirus, and porcine parainfluenza virus. Dyodelta-PV was first identified in a skin sample from a normal pig and is not considered a significant pathogen of swine.[9] Porcine parainfluenza virus (PPIV) is linked to respiratory diseases in swine.[12] Though uncommon, the presence of PPIV along with PRRSV and other respiratory pathogens can contribute to more severe disease. Overall, although the viruses identified in oral fluid may have only a limited impact on swine health, they can function as disease co-factors in pigs that are immunosuppressed as a result of PRRSV or PCV2.

As summarized in Table 5, several bacteria were identified in the oral fluid samples. Some are linked with PRRSV infection; function as cofactors to cause more severe disease. [4] Examples include *M. hyponeumoniae* (identified in serum), *P. multocida, A. pleuropneumoniae,* and *S, suis* are co-factors linked to PRDC. Signatures for *P. multocida* and *A. pleuropneumoniae* were also present in tonsil. Molecular signatures for *S. suis* were found in all oral fluid samples. PRRSV has been shown to increase susceptibility of pigs to diseases caused by *S. suis.* Nursery pigs inoculated with PRRSV followed by *S. suis* develop moderate to severe respiratory disease, mild to severe lameness with associated joint effusion, and have increased mortality.[13,14] The remaining bacteria are considered normal flora or opportunistic; present on surfaces in the upper respiratory tract, oral cavity, epidermis, or present in feces.[17]

The last sample evaluated in this study was tonsil, a tissue that is sampled by scraping, swabbing or removal at the time of necropsy. Similar to the results obtained for serum and oral fluid, PCV2 was easily detected. The inability to detect PRRSV was the result of low amounts of virus at the time of sacrifice (see Figure 1). TTSuV and porcine oncovirus signatures were also detected.

Besides the association of a given microbe with pigs, one way to evaluate the validity of a result is to determine the number of targets recognized in the array. A summary for the all of the microbes detected in this study is presented in Table 6. The results show that multiple probe regions across the genome were detected for each microbe. Between 82% and 100% of expected probes showed a positive result, providing highly confident detection. The Table also shows that oral fluid provided the

greatest number of results, including the presence of several microbes associated with PRRSV and PCV2.

This study demonstrates the utility in the use of Lawrence Livermore Microbial Detection Array in routine clinical diagnostics and surveillance. The study also demonstrates the unique properties of each clinical sample. Even though serum is widely used in molecular diagnostics, oral fluid may emerge as the sample of choice for conducting routine herd surveillance. However, our results indicate it should be used with caution, as the important swine pathogen PRRS is present at levels too low to be detected by either PCR or microarray even near the times of highest viremia during the course of infection.

Declaration of conflicting interests

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Table 1. Pathogen microarray analysis of PCV2 and PRRSV preparations used for infection of pigs.

Inoculum	Source	Viruses and bacteria detected by the array
PCV2	Lymph node suspension	PCV2
		TTSuV* 1a
		TTSuV 1b
		TTSuV k2b
		Porcine type-C oncovirus, endogenous retrovirus A
		Porcine type-C oncovirus, endogenous retrovirus E
PRRSV	MARC-145 cells	PRRSV
		Mason-Pfizer simian retrovirus

*Torque teno sus virus (TTSuV).

Table 2. Detection of PCV2 and PRRSV in serum by PCR and pathogen microarray.

			PCV2*			**PRRSV***				
Sample	Pig	Day†	Ct	Copies	Array‡	Ct	Copies	Array‡	Viruses§	Bacteria¦
145	13	13	14.2	6.8	Y	20.2	5.6	Y	A,D,E	
146	215	13	13.6	7.5	Y	21.9	5.1	Y	A,B,C,D,E	
147	89	13	16.5	6.1	Y	29.2	3.0	Y	B,D,E,F	
148	131	13	20.1	5.2	Y	30.3	2.8	Y	B,D,E	
149	41	13	35.6	1.0	N	21.9	5.1	Y	D,E	
150	79	13	36.1	1.0	N	22.2	5.0	Y	A,D,E	
157	97	21	22.2	5.0	Y	>40	<1	N	D,E	B
158	234	21	28.2	3.4	Y	27.2	3.5	Y	A,B,C,D,E	
159	219	21	20.8	5.4	Y	31.5	2.3	N	D,E	C
160	17	21	25.2	4.3	Y	25.8	3.9	Y	D,E	A
161	173	21	>40	<1	N	32.4	2.0	N	A,B,D,E	A,C
162	104	21	17.1	6.5	Y	30.6	2.6	N	A,B,D,E	
163	197	21	18.5	6.1	Y	28.0	3.4	Y	B,D,E	
164	118	21	21.1	5.4	Y	29.0	3.0	Y	B,D	
165	237	21	19.2	5.9	Y	26.2	3.9	Y	B,C,D	A
166	199	21	17.3	6.5	Y	34.3	1.5	N	B,C,D,E	
167	105	21	15.9	6.8	Y	25.0	4.3	Y	B,D,E,	
168	258	21	17.4	4.7	Y	28.1	3.3	Y	B,D,E	C

*Results show PCR Ct value and copies reported as the log template number per reaction.
†Day after dual challenge with PRRSV and PCV2
‡Key for array detection; Y = Yes; N = No
§Additional viruses detected in serum at a 99% confidence level. Key: A, torque teno sus virus (TTSuV) 1a; B, TTSuV 1b; C, TTSuV K2b; D, porcine type-C oncovirus retrovirus A; E, porcine type-C oncovirus retrovirus E; F, porcine bocavirus 4-1
¦ Additional bacteria key: A, *Delftia acidovorans*; B, *Mycoplasma hyopneumoniae*; C, *Stenotrophomonas maltophilia*

Table 3. PCR and microarray detection of viruses in oral fluid samples at 13 days after infection with PRRSV and PCV2.

		PCV2*			PRRSV*			Additional
Sample	Pen†	Ct	Copies	Array‡	Ct	Copies	Array‡	Viruses§
151	13	21.1	5.6	Y	>40	<1	N	A,C,E
152	15	22.6	5.2	Y	>40	<1	N	A,C
153	19	21.7	5.4	Y	38.6	<1	N	B,C
154	18	23.3	4.9	Y	>40	<1	N	A,C
155	21	25.3	4.3	Y	>40	<1	N	A,C,F
156	22	23.8	4.8	Y	>40	<1	N	B,C,D

*Results show PCR Ct value and copies reported as the log template number per reaction.
†Samples were collected from approximately 10 pigs per pen
‡Key for array detection; Y = Yes; N = No
§Key: A, TTSuV k2b; B, TTSuV 1b; C, porcine stool-associated circular virus; D, porcine astrovirus WBAstV−1; E, dyodelta-papillomavirus 1 (sus scrofa papillomavirus); F, porcine parainfluenza virus

Table 4. Microarray detection of bacteria in oral fluid samples at 13 days after infection with PRRSV and PCV2.

Sample	A*	B	C	D	E	F	G	H	I	J	K	L	M
151	+	+	+	+	+	+	+	-	+	-	-	-	-
152	+	+	+	+	+	-	+	-	-	-	+	-	-
153	+	+	+	+	+	-	-	+	-	-	-	-	-
154	+	+	+	+	-	-	-	+	-	-	+	+	-
155	+	+	+	-	-	+	+	-	-	+	-	+	-
156	+	+	+	-	-	+	-	-	+	+	-	-	+

*Key: A, *Streptococcus suis*; B, *Clostridium sp*; C, *Staphylococcus sp*; D, *Enterococcus sp*; E, *Psychrobacter sp*; F, *Aerococcus viridans*; G, *Bibersteinia trehalosi*; H, *Actinobacillus pleuropneumoniae*; I, *Lactococcus sp*; J, *Mycoplasma moatsii*; K, *Bergeyella zoohelcum*; L, *Enhydrobacter aerosaccus*; M, *Lactobacillus sp*

Table 5. PCR and microarray analysis of tonsils at 42 days after infection of pigs with PCV2 and PRRSV.

		PCV2 Serum*		PCV2 Tonsil	PRRSV Serum*		PRRSV Tonsil		
Sample	**Pig**	**Ct**	**Copies**	**Array‡**	**Ct**	**Copies**	**Array‡**	**Viruses§**	**Bacteria¦**
169	97	ND†	ND	Y	ND	ND	N	D	
170	234	30.6	6.1	Y	>40	<1	N	D	
171	219	34.4	1.6	N	39.2	<1	N	D	
172	17	30.9	2.8	Y	>40	<1	N	D	
173	173	32.2	2.4	Y	>40	<1	N	D	
174	104	23.2	4.9	Y	36.0	0.9	N	D	
175	197	ND	ND	Y	ND	ND	N	B,C,D	
176	118	ND	ND	Y	ND	ND	N	D	
177	237#	ND	ND	Y	ND	ND	N	A,D	A,B
178	199#	ND	ND	Y	ND	ND	N	C,D	A,C
179	105#	ND	ND	Y	ND	ND	N	D	
180	258	23.1	3.9	Y	39.0	<1	N	D	

*Results show PCR Ct value and copies reported as the log template number per reaction.
†ND, not determined
‡Key for array detection; Y = Yes; N = No
§Additional viruses, key: A, TTSuV 1a; B, TTSuV 1b; C, TTSuV K2b; D, Porcine type−C oncovirus A
¦Additional bacteria, key: A, *Actinobacillus pleuropneumoniae*; B, *Pasteurella multocida*; C, *Pasteurella aerogenes*
#Pig removed from study at 35 days due to morbidity.

Table 6. Summary of infectious agents detected by the pathogen microarray. Samples sources are: Inoculum (I), serum (S), oral fluid (O), tonsil (T).

Organism	Source	Probes Detected (% detected)
Viruses		
PCV2	I,S,O,T	????
PRRSV	I,S	????
Simian retrovirus	I	????
TTSuV	I,S,O,T	34/34 (100%)
Porcine ERV A	I,S,T	32/32 (100)
Porcine ERV E	I,S,T	18/18 (100)
Porcine bocavirus	S	18/22 (82)
Porcine stool-associated virus	O	20/20 (100)
Astrovirus	O	17/19 (89)
Dyodelta-papillomavirus	O	18/18 (100)
Porcine parainfluenza virus	O	18/19 (95)
Bacteria		
Aerococcus viridans	O	23/23 (100)
Actinobacillus pleuropneumoniae	O	64/65 (98)
Bergeyella zoohelcum	O	28/33 (85)
Bibersteinia trehalosi	O	48/61 (79)
Clostridium sp.	O	36/40 (90)
Enhydrobacter aerosaccus	O	24/26 (92)
Enterococcus sp.	O	67/73 (92)
Lactococcus sp	O	108/115 (94)
Mycoplasma sp.	O,S	32/36 (89)
Pasteurella multocida	O,T	17/18 (94)
Pasteurella aerogenes	T	xx/xx(xx)
Psychrobacter sp.	O	41/50 (82)
Staphylococcus sp.	O	60/68 (88)
Stenotrophomonas maltophilia	S	9/9 (100)
Streptococcus sp.	O	147/147 (100)

Figure legend

Figure 1. PRRSV and PCV2 infection in experimentally infected pigs. Mean viremia for PRRSV are shown in open circles and PCV2 in solid circles. Data from 200 pigs.